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Directed Genomic Integration, Gene Replacement, and Integrative Gene Expression in *Streptococcus thermophilus*

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Several pGEM5- and pUC19-derived plasmids containing a selectable erythromycin resistance marker were integrated into the chromosome of *Streptococcus thermophilus* at the loci of the lactose-metabolizing genes. Integration occurred via homologous recombination and resulted in cointegrates between plasmid and genome, flanked by the homologous DNA used for integration. Selective pressure on the plasmid-located erythromycin resistance gene resulted in multiple amplifications of the integrated plasmid. Release of this selective pressure, however, gave way to homologous resolution of the cointegrate structures. By integration and subsequent resolution, we were able to replace the chromosomal *lacZ* gene with a modified copy carrying an in vitro-generated deletion. In the same way, we integrated a promoterless chloramphenicol acetyltransferase (*cat*) gene between the chromosomal *lacS* and *lacZ* genes of the lactose operon. The inserted *cat* gene became a functional part of the operon and was expressed and regulated accordingly. Selective pressure on the essential *lacS* and *lacZ* genes under normal growth conditions in milk ensures the maintenance and expression of the integrated gene. As there are only minimal repeated DNA sequences (an *NdeI* site) flanking the inserted *cat* gene, it was stably maintained even in the absence of lactose, i.e., when grown on sucrose or glucose. The methodology represents a stable system in which to express and regulate foreign genes in *S. thermophilus*, which could qualify in the future for an application with food.

Streptococcus salivarius subsp. *thermophilus* (*S. thermophilus*), a homofermentative, thermophilic lactic acid bacterium, is an important microorganism for the fermentation of food. It is predominantly used in starter cultures for the production of fermented milk products such as Swiss and Italian cheese and yogurt. Despite its widespread and long-time use in fermentation processes, progress in the genetics of this organism has been made only recently. Some genes have been isolated and analyzed (2, 16, 19, 32, 33, 41, 50). Among these are the *lacS* (lactose permease) and *lacZ* (β-galactosidase) genes, which are separated by only three nucleotides and are probably translationally coupled (32).

Several gene transfer techniques such as conjugation (12, 37), transfection (27), and transformation (28, 45) have been reported for *S. thermophilus*. These techniques enable the examination and use of already existing bacterial plasmids as cloning vectors as well as a beginning in designing new vector systems based on cryptic, homogenic plasmids (13, 15, 26, 44, 46). Selection applied for ensuring plasmid uptake and maintenance make in most cases use of marker genes conferring resistance to antibiotics. Although very convenient for laboratory-scale experiments, such selection systems cannot be applied to food production. To date, no food-grade gene transfer and expression system for *S. thermophilus* has been reported.

Plasmids are not a priori segregated in a stable way and may be lost under nonselective growth conditions. This in particular may be true for plasmid systems which are genetically engineered and carry heterologous DNA. In order to stabilize plasmids, they can be integrated into the bacterial genome via a single, homologous recombination process. So far, this has been reported for several different bacterial species, e.g., *Escherichia coli* (14, 34, 36), *Bacillus subtilis* (8, 30), *Lactococcus lactis* (5, 20), and others (24). For *S. thermophilus*, genomic integration has been mentioned to work (25, 25a). Such integration of plasmids usually results in two direct repeats of homologous DNA flanking the integrated plasmid, a structure which is similar to insertion sequence-mediated cointegrates (18). Because of the direct repeats, integrated plasmids can be excised and lost again or upon proper selection be tandemly amplified. The integration technique is a very versatile tool for genetic manipulation and can be used to inactivate, replace, or integrate genes and to genetically tag specific genes (40).

It was the aim of our study to develop a system for genomic plasmid integration in *S. thermophilus* and to demonstrate its use for gene disruption, heterologous gene integration, and expression. The final constructions should be, without taking the foreign gene being integrated and expressed into consideration, food grade. This means that no heterologous DNA from plasmids or marker genes should be present in the bacterial cell. Furthermore, integration of the gene should be such as to guarantee genetic stability and expression.

In order to achieve genomic integration in *S. thermophilus*, we made use of an optimized transformation system (23) and used *E. coli*-based plasmids which do not autonomously replicate in *S. thermophilus*. As a target region for integration, we chose the lactose operon, consisting of the *lacS* and *lacZ* genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. thermophilus* ST11, a starter strain for yogurt production, is from our collection. The *E. coli* strains used were BZ234 (collection from the Biozenter, University of Basel, Basel, Switzerland) and JM108 (49). The plasmids used were pVA838 (21), pNZ12 (43), pGEM-5Zf (Promega, Madison, Wis.), and pUC18

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TABLE 1. Plasmids used

Plasmid	Selection marker(s)	Description	Reference or source
pVA838	Erm		21
pNZ12	Cat		43
pGEM5-838	Amp, Erm	pGEM-5Zf(-)erm_{pVA838}	D. Pridmore
pUC-838	Amp, Erm	pUC19erm_{pVA838}	B. Suri
pDP211	Amp, LacZ	pUC19$lacZ_{ST11}$	D. Pridmore
pDP222	Amp	pDP211$\Delta lacZ_{BglII-BglII}$	D. Pridmore
pDP225	Amp	pDP211$\Delta lacZ_{HpaI-HpaI}$	D. Pridmore
pDP228	Amp	pGEM-5Zf(-)*PstI-SpeI* (Fig. 1)	D. Pridmore

(49). Plasmids pUC-838 and pGEM5-838 contain the 1.7-kb *Hin*dIII-*Ava*I fragment (blunt ended) carrying the erythromycin resistance gene (Em^r) of pVA838 in their unique *Sma*I sites of pUC18 and pGEM-5Zf, respectively. pDP211 contains the 7.0-kb *Pst*I fragment carrying the *lacZ* gene of ST11 in the unique *Pst*I site of pUC19 (49) (construction similar to that of pRH116 [16]). pDP222 and pDP225 are derivatives of pDP211 having the *lacZ* internal 1.3-kb *Bgl*II and 1.0-kb *Hpa*I fragments, respectively, deleted (Table 1). pDP228 is pGEM-5Zf containing the 2.4-kb *Pst*I-*Spe*I fragment of pDP211 (Fig. 1).

Media. *S. thermophilus* was grown in HJ broth (3% tryptone, 1% yeast extract, 0.5% KH_2PO_4, 0.5% beef extract) supplemented with either 1% lactose and/or glucose (17), M17 broth (Difco Laboratories, Detroit, Mich.) or MSK broth (9% reconstituted skim milk powder, 0.1% yeast extract) at 42°C. For the transport assay, cells were grown in Elliker broth (10) containing 0.5% beef extract and lactose, sucrose, and/or galactose (final concentrations, 20 mM each). *E. coli* strains were grown in LB (0.5% NaCl, 1% tryptone, 1% yeast extract) at 37°C. Media were solidified for plating by the addition of 1.5% agar. Erythromycin, chloramphenicol, ampicillin, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were added individually as indicated.

Transformation of *S. thermophilus*. *S. thermophilus* ST11 was grown in HJ broth supplemented with glucose and lactose overnight at 42°C. The next day, 33 ml of fresh medium was inoculated with 700 μl of the overnight culture and grown at 42°C for another one to three generations to a maximal optical density at 600 nm of 0.3. The cells were harvested by centrifugation (5 min at 2,000 × *g*), washed once with 5 mM potassium phosphate (pH 7.0), resuspended gently in freshly prepared ice-cold EPM (0.3 M raffinose, 5 mM potassium phosphate [pH 6.1], 0.5 mM $MgCl_2$) to an optical density at 600 nm of 0.9, and kept on ice at 0°C. Two hundred microliters of this cell suspension was added to a prechilled 0.2-cm electroporation cuvette containing plasmid DNA and electroporated with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) at 25 F, 400 Ω, and 2.05 kV. Immediately after pulsing, 1 ml of 1.2× concentrated M17 broth, supplemented with sucrose, was added to the cuvette. The suspension was mixed, transferred to a sterile tube, and incubated for 4 h at 42°C. Four milliliters of melted soft agar (M17 broth supplemented with 1% sucrose and 0.6% agar) was added to each of the cultures, and the mixtures were plated on M17 agar plates containing 1% sucrose and 2.5 μg of either erythromycin or chloramphenicol per ml. The plates were incubated at 42°C for 2 to 3 days under anaerobic conditions (BBL GasPak; Becton Dickinson and Co.).

Construction of plasmids. The *Pst*I-*Spe*I fragments from pDP222 and pDP225, containing the *lacZ* gene with an

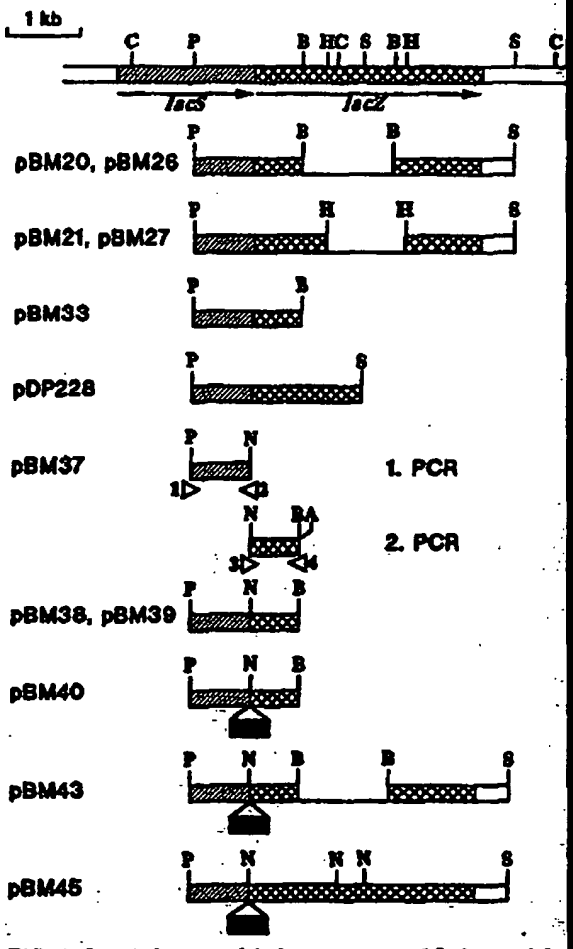

FIG. 1. Restriction map of the lactose operon of *S. thermophilus* and schematic representation of the constructed plasmids. Hatched boxes indicate the lactose permease gene and cross-hatched boxes indicate the β-galactosidase gene, denoted as *lacS* and *lacZ*, respectively. Arrows indicate the orientations of the genes. Abbreviations for restriction sites: A, *Sac*I; B, *Bgl*II; C, *Cla*I; H, *Hpa*I; N, *Nde*I; P, *Pst*I; S, *Spe*I. Labelled triangles indicate the locations and orientations of oligonucleotide primers used for PCR. The solid boxes represent the *cat* gene.

internal deletion, were ligated into vector pGEM5-838 at the unique *Pst*I and *Spe*I sites and transformed to *E. coli* BZ234. Transformants were grown on LB-erythromycin (1-mg/ml) plates. Correct clones were identified by restriction analysis and named pBM20 and pBM21, respectively. To remove the ampicillin resistance gene, pBM20 and pBM21 were digested with *Fsp*I, which cuts at positions 1617 and 2843 of pGEM-5Zf (Promega), religated, and transformed to BZ234. Selection was done on LB-erythromycin plates as described above. Plasmids having the correct 1.2-kb *Fsp*I deletion were named pBM26 and pBM27, respectively (Fig. 1). Vector pUC-838 was digested with *Fsp*I, religated, and transformed to BZ234 as described above. The resulting plasmid was linearized at its unique *Eco*RI site, blunt ended by a filling-in reaction (22), ligated, and proliferated in BZ234. The new vector was opened at its unique *Pst*I and *Bam*HI sites and ligated to the agarose gel-purified 1.55-kb *Pst*I-*Bgl*II fragment of pBM20. The ligation mixture was transformed to BZ234, and transformants were selected on LB-erythromycin plates. Correct clones were named pBM33 (Fig. 1).

In order to insert a cloning site precisely between the two *lac* genes, polymerase chain reaction (PCR) amplification of *Fsp*I-linearized pDP228 was performed with the synthetic oligonucleotides 5'-GGTTTTCCCAGTCACGAC (primer 1) and 5'-GTCATGTTCATATGTTATTCTCCTTT (primer 2, introducing an *Nde*I site). The amplified fragment was digested with *Pst*I and *Nde*I, ligated into the *Pst*I- and *Nde*I-digested vector pGEM-5Zf, and transformed to BZ234, and the cells were selected for growth on LB-ampicillin (100-μg/ml) plates. Correct clones carrying the 900-bp fragment were named pBM37. A second PCR was carried out with linearized pDP228 and the oligonucleotides 5'-AAAGGAGAAT AACATATGAACATGAC (primer 3) and 5'-TTGGGAGCT CTCCCTTAACAAAGAGA (primer 4, containing a *Sac*I site next to the *Bgl*II site). The ca. 700-bp amplified fragment was digested with *Sac*I and *Nde*I and ligated into *Sac*I- and *Nde*I-digested pBM37. The ligation mixture was transformed to BZ234, and the cells were grown on LB-ampicillin (100-μg/ml) plates. The resulting plasmid was named pBM38 (Fig. 1). Plasmid pBM38 was digested with *Bst*EII and *Dra*III, and the resulting 650-bp fragment, containing the new junction between the *lacS* and *lacZ* genes, was isolated from an agarose gel. Similarly, pBM33 was digested with *Bst*EII and *Dra*III, and the fragment containing the vector backbone was isolated and ligated to the 650-bp fragment from pBM38. The ligation mixture was transformed to BZ234, plated onto LB-erythromycin (1-mg/ml) plates, and incubated at 37°C. Correct clones carrying the inserted *Nde*I site originating from pBM38 were identified as pBM39 (Fig. 1).

To insert the *cat* gene from pNZ12, originally derived from pC194 (9), into the *Nde*I site, the *cat* gene was PCR amplified with the synthetic oligonucleotides 5'-ATATCATATGAA CTTTAATAAAATTGAT and 5'-ATTATCATATGTTATA AAAGCCAGTCATTAG as primers to generate *Nde*I sites at the start and end of the gene. The ca. 670-bp PCR-amplified fragment was digested with *Nde*I and ligated into the unique *Nde*I site of pBM39. The ligation mixture was transformed to BZ234, plated onto LB-erythromycin (1-mg/ml) plates, and incubated at 37°C. The plasmid contents of individual colonies were analyzed, and clones having the *Nde*I fragment inserted in the correct orientation, i.e., the *cat* gene reading in the same direction as *lacS* and *lacZ*, were identified and named pBM40 (Fig. 1).

In order to obtain a higher frequency of homologous genomic integration upon transformation, the construct with the inserted *cat* gene was manipulated further to generate a plasmid containing the entire *lacZ* sequence. For that purpose, pBM40 was digested with *Bst*EII and *Dra*III, and the 1.3-kb fragment containing *cat* was isolated by agarose gel electrophoresis. Similarly, pBM26 was digested with *Bst*EII and *Dra*III, and the fragment containing the vector backbone was isolated. The two isolated fragments were ligated and transformed to BZ234, and the cells were grown on LB-erythromycin (1-mg/ml) plates. Correct clones were named pBM43 (Fig. 1). To reconstitute the complete *lacZ* gene on pBM43, the 1.3-kb *lacZ* internal *Bgl*II fragment isolated from plasmid pDP211 was ligated into pBM43, which was first linearized at its *Bgl*II site and treated with alkaline phosphatase. The ligation mixture was transformed to JM108 and plated onto LB-erythromycin plates containing 40 μg of X-Gal per ml. After incubation, the plasmid contents of individual blue colonies were isolated and analyzed. Correct clones carrying the entire *lacZ* gene were named pBM45 (Fig. 1).

DNA manipulations. Plasmid DNA from *E. coli* was isolated and when needed was purified on CsCl gradients by the method of Maniatis et al. (22). Agarose gel electrophoresis, restriction enzyme digestions, ligations, and transformation of *E. coli* were performed by standard procedures (22). Genomic DNA of *S. thermophilus* was isolated as reported for lactobacilli (7) and stored at 4°C. Southern blot hybridizations were performed as described by Southern (47). DNA probes were ^{32}P labelled by the random priming method (22). Hybridization and washing of the blots were performed under stringent conditions. PCR amplification was done by the method of Saiki et al. (38, 39).

Transport and enzymatic assays. Cells were cultivated microaerobically in Elliker broth at 42°C and harvested at an optical density at 660 nm of 0.6 to 0.7, unless otherwise indicated. They were washed twice and resuspended in 50 mM potassium phosphate (pH 6.5) supplemented with 2 mM $MgSO_4$. Methyl-1-thio-β-D-galactopyranoside (TMG)/[^{14}C]TMG counterflow activity was assayed by the filtration method as described previously (11). The final [^{14}C]TMG concentration in the transport assay was 48 μM. The assay temperature was 23°C.

Cell-free lysates were prepared by cycle sonication (eight times for 15 s each, with intervals of 45 s) at 4°C under nitrogen with a macrotip at an output of 4 μm. The sonicated suspensions were centrifuged for 1 h at 48,000 × *g*, and the supernatants, containing 5 to 10 mg of total protein per ml, were used for estimating the enzymatic activities. β-Galactosidase was assayed at 37°C by the method of Miller (29), and the chloramphenicol acetyltransferase activity was assayed as described by Shaw (42). Total protein concentrations were estimated by a dye binding assay (3).

RESULTS

Transformation and genomic integration. The plasmids as listed in Table 2 were used to transform ST11. Selection for transformants was on M17 agar-sucrose plates containing 2.5 μg of either erythromycin or chloramphenicol per ml. As indicated, plasmids were transformed either as covalently closed circular DNA or linearized at their unique *Pst*I sites (Table 2).

The frequency of transformation with pVA838 and pNZ12 corresponded to the expected values (23). The *E. coli* vectors pGEM5-838 and pUC-838 were not able to replicate or integrate in ST11; hence, no erythromycin-resistant col-

TABLE 2. Transformation frequencies

Plasmid	Selection marker	Size (kb) of:		No. of transformants per μg of DNA
		Plasmid	Homologies	
pVA838	Erm[a]	9.2		1.3×10^4
pNZ12	Cm[b]	4.3		1.8×10^3
pGEM5-838	Erm	4.7		<0.1
pUC-838	Erm	4.4		<0.1
pBM20	Erm	7.8	1.55/1.60	24
pBM20 lin.[c]	Erm	7.8	1.55/1.60	140
pBM21	Erm	8.1	1.91/1.49	33
pBM21 lin.	Erm	8.1	1.91/1.49	150
pBM26	Erm	6.6	1.55/1.60	24
pBM26 lin.	Erm	6.6	1.55/1.60	250
pBM27	Erm	6.9	1.91/1.49	25
pBM27 lin.	Erm	6.9	1.91/1.49	125
pBM33	Erm	4.9	1.55	5
pBM33 lin.	Erm	4.9	1.55	250
pBM40	Erm	5.7	0.88/0.67	10
pBM40 lin.	Erm	5.7	0.88/0.67	100
pBM45	Erm	8.5	0.88/3.56	41
pBM45 lin.	Erm	8.5	0.88/3.56	240

[a] 2.5 μg of erythromycin per ml.
[b] 2.5 μg of chloramphenicol per ml.
[c] lin., linearized at the unique *Pst*I site.

onies were obtained in several transformation trials. Transformation with *E. coli* vectors carrying short stretches of genomic DNA of the ST11 *lac* region resulted in erythromycin-resistant transformants at a lower frequency. Upon linearization of the plasmids at their unique *Pst*I sites, located at the junction between vector and *lac* operon DNA, the number of erythromycin-resistant transformants per μg of DNA was significantly increased.

Analysis of transformants. Independent erythromycin-resistant transformants from pBM20 and pBM21 were isolated and cultivated in M17 broth with 2.5 μg of erythromycin per ml under anaerobic conditions at 42°C. Genomic DNA of the cultures was extracted and their *Pst*I digests were analyzed by Southern blot hybridizations with pBM20 as the labelled DNA probe (Fig. 2A). For most of the transformants, the chromosomal 7-kb *Pst*I fragment carrying the *lacZ* gene of ST11 was lost and two new fragments of 9 and 6 kb appeared. This clearly indicates that the plasmids were integrated into the genome within the homologous *lacZ* region. The disappearance of the 7-kb *Pst*I fragment further discloses that the integration event placed the imported *lacZ* region, containing the in vitro-generated deletion downstream of the original, wild-type *lacZ* gene (Fig. 3). In one case, however, the presence of the 7-kb fragment points to an event which placed the integrated plasmid upstream of the original *lacZ* gene (Fig. 2A, lane 5). Several transformants possessed an additional *Pst*I fragment at ca. 8 kb, which corresponds in size to pBM20 and pBM21. Since these two plasmids are unable to replicate autonomously in ST11, the presence of the 8-kb fragment points to amplification of the genomic integrated plasmids (Fig. 3). As can be further suggested from the variable hybridization intensities of the different bands, multiple amplifications of the integrated plasmids, flanked by either the integrated or wild-type *lacZ* region, have occurred in some cases. Further hybridization experiments with *Hind*III and *Eco*RI genomic digests and probing of the hybridization filters with only either pGEM5-838 or *lacZ* DNA confirmed the above-described results (data not shown).

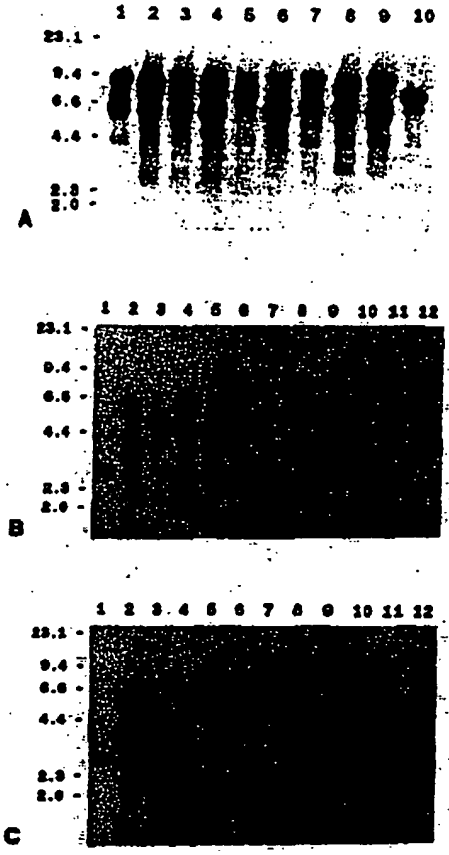

FIG. 2. Southern blots of total genomic DNA of erythromycin-resistant transformants. Plasmids used for transformation were pBM20 (A, lanes 1 to 5), pBM21 (A, lanes 6 to 9), pBM26 (B, lanes 2 to 6 and 8 to 12), and pBM27 (C, lanes 2 to 6 and 8 to 12). Untransformed ST11 is shown in lane 10 of panel A, lanes 1 and 7 of panel B, and lanes 1 and 7 of panel C. Endonuclease digestion of total genomic DNA was with *Pst*I for all lanes of panel A, lanes 7 to 12 of panel B, and lanes 7 to 12 of panel C and with *Cla*I for lanes 1 to 6 of panel B and lanes 1 to 6 of panel C. ^{32}P-labelled pBM20 (A), pBM26 (B), and pBM27 (C) were used as DNA probes for hybridization. Size markers are indicated in kilobases.

Similar results were obtained from Southern blot analysis performed with *Pst*I- and *Cla*I-digested genomic DNA of erythromycin-resistant transformants obtained with pBM26 and pBM27 (Fig. 2B and C) and pBM33 (data not shown). The absence of the 3.1-kb *Cla*I fragment in all transformants clearly demonstrates that the plasmids were integrated into the genome at their homologous locus, i.e., within the above-mentioned *Cla*I fragment. Again, variable amplifications of the inserted pBM26 were observed, and some of them are schematically depicted in Fig. 4. Interestingly, amplifications with pBM27 were less frequent than with pBM26.

Plasmid amplifications. The degree of amplification was with the smallest plasmid, pBM33, investigated. Genomic DNA isolated from erythromycin-resistant transformants was digested with *Eco*RV, which does not cut within the plasmid (or the homologous *lacZ* region), and analyzed by Southern analysis with pBM33 as the probe (Fig. 5). The

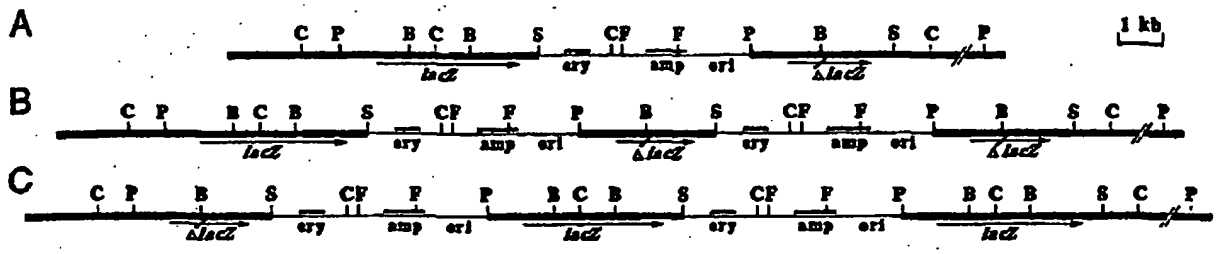

FIG. 3. Restriction maps of genomic pBM20 cointegrates. (A) Structure of the isolate from Fig. 2A, lane 1; (B) structure of the isolate from Fig. 2A, lane 3; (C) structure of the isolate from Fig. 2A, lane 5. Thick lines represent *S. thermophilus* DNA, and thin lines represent DNA of another origin. The *lacZ* gene and its deletion derivatives (Δ*lacZ*) are indicated by arrows. The erythromycin (ery) and ampicillin (amp) resistance genes and the *E. coli* origin of replication (ori) are indicated. Abbreviations for restriction sites: B, *Bgl*II; C, *Cla*I; F, *Fsp*I; P, *Pst*I; S, *Spe*I.

2.6-kb *Eco*RV fragment from ST11 containing the homologous *lacZ* region was increased in size in the transformants upon integration and subsequent amplification. Fragment sizes varied from 12 to 17, 22, and 27 kb, corresponding with two, three, four, and five copies of integrated pBM33, respectively. A heterogeneity in copy number of the amplifications could be observed among as well as within cell cultures.

Resolution of the integrated plasmids. The integrated plasmids were flanked by homologous *lacZ* sequences (Fig. 3). Through homologous recombination, these flanking sequences probably initiated the observed amplification processes. In a similar way, homologous recombination may eliminate the integrated plasmids from the genome (Fig. 6). Depending on which part of the homologous sequences is used for this recombination process, either a single copy of the wild-type or the integrated version of the *lacZ* region, i.e., the *Bgl*II-*Bgl*II or *Hpa*I-*Hpa*I deletion from pBM20 or pBM21, respectively, would be retained on the bacterial genome. In the latter case, the *lacZ* gene would be disrupted. The cells would not be able to ferment lactose and would grow as white colonies on appropriate X-Gal agar plates (29).

To resolve the integrants, several of the above-described pBM20 and pBM21 erythromycin-resistant transformants were cultivated for 40 to 45 generations in HJ broth supplemented with glucose but lacking erythromycin. Cell cultures were diluted and plated onto HJ-glucose agar plates containing 25 μg of X-Gal per ml. In most cases, white colonies appeared among a majority of blue colonies (1 to 10% white colonies). Individual white colonies were isolated and purified by restreaking them onto fresh X-Gal agar plates. The purified colonies were tested for growth on agar plates containing erythromycin. No growth was observed, indicating that the erythromycin resistance gene and the integrated plasmid were evicted.

Analysis of resolved transformants. Genomic DNA of resolved erythromycin-sensitive transformants was digested with *Pst*I and *Hin*dIII and analyzed by Southern analysis with pBM20 as the labelled DNA probe (Fig. 7). The results showed that the 7-kb *Pst*I and 3.1-kb *Hin*dIII fragments of ST11 were reduced in size for the resolved transformants according to the extent of the in vitro-generated deletions in the *lacZ* region of pBM20 and pBM21, respectively. Furthermore, hybridization bands identifying the integrated plas-

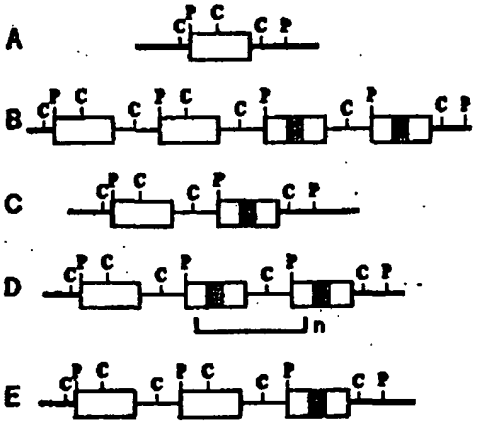

FIG. 4. Schematic representation of genomic pBM26 cointegrate DNA structures. (A) Structure of wild-type ST11 DNA when analyzed by *Cla*I and *Pst*I restriction. The box represents the homologous stretch of DNA used for plasmid integration and extends from the *lacS* internal *Pst*I to the *Spe*I site downstream of *lacZ* (Fig. 1). Cointegrate structures of the isolate from Fig. 2B, lanes 2 and 8 (B), lanes 3 and 9 (C), lanes 4 and 10 (D), and lanes 5 and 11 (E) are also shown. Hatched areas indicate the in vitro-generated *Bgl*II-*Bgl*II deletion. Thin lines are integrated *E. coli* plasmids. The bracket underneath map D points to a further amplification. Its copy number (n) could not be determined accurately. Abbreviations for restriction sites: P, *Pst*I; C, *Cla*I.

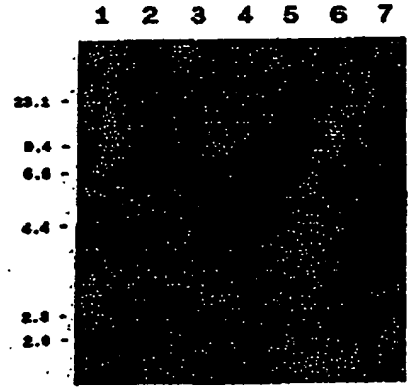

FIG. 5. Southern blot of genomic integrated pBM33. Lane 1 is ST11; lanes 2 to 7 are independent erythromycin-resistant transformants of pBM33. Genomic DNA was digested with *Eco*RV and probed with ^{32}P-labelled pBM33. Size markers are indicated in kilobases.

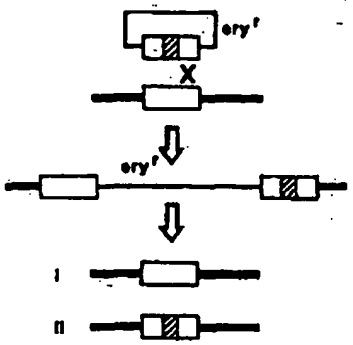

FIG. 6. Schematic representation of gene replacement via plasmid integration and subsequent resolution. Boxes represent the homologous stretch of DNA susceptible to recombination. Hatched areas indicate the in vitro-generated deletion (for pBM20 and pBM21) or the inserted *cat* gene (for pBM45). Thin lines are plasmid DNA, and thick lines ST11 genomic DNA. The erythromycin resistance gene (ery') is indicated. The two possible resolution products are labelled i and ii.

mid, i.e., the 9-kb *PstI* and 5.5-kb *HindIII* fragments, were absent. Southern blot analysis with pUC-838 as the probe showed no hybridization with the resolved transformants, indicating the physical absence of plasmid backbone sequences (data not shown). These results clearly demonstrate that the plasmid cointegrate structures were resolved and that the wild-type *lacZ* region was replaced with the imported copy from pBM20 and pBM21, respectively.

Integration of *cat* into the lactose operon. In *S. thermophilus*, the lactose permease (*lacS*) and β-galactosidase (*lacZ*) genes are located on the bacterial genome and are separated by only three nucleotides (32). Constructs were made to integrate the *cat* gene into this operon, between the *lacS* and *lacZ* genes. To conserve translational coupling of the genes, they were all separated by 3 bp each (Fig. 8). Selective pressure on expression of the *lac* genes, i.e., growth on lactose as the sole carbohydrate source, should then ensure expression of the integrated *cat* gene.

To make the appropriate constructions for *cat* integration, an *NdeI* site was first engineered in front of the *lacZ* gene, overlapping with its ATG start codon, on plasmid pBM33, resulting in pBM39 (Fig. 1). The spacing of the two genes remained intact, and the sequence alterations were restricted to the intergenic region. The *cat* gene was PCR amplified from pNZ12 with primers that introduce *NdeI* sites at the start codon and immediately downstream of the stop codon of the gene. The amplified and *NdeI*-digested fragment was inserted into the *NdeI* site of pBM39, between the *lacS* and *lacZ* genes, resulting in pBM40 (Fig. 1). To ensure efficient genomic integration, pBM45, which has a longer stretch of homologous DNA for recombination with the genome, was constructed (Fig. 1).

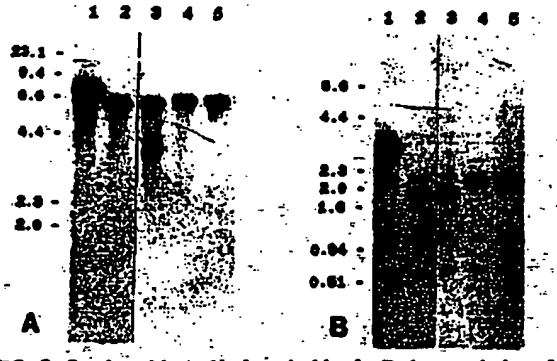

FIG. 7. Southern blots of isolates lacking *lacZ* after resolution of plasmid cointegrates. Shown are *PstI* (A) and *HindIII* (B) digests of total genomic DNA. ST11 (lane 1) and isolates with resolved cointegrate structures derived from pBM20 (1.3-kb *BglII* deletion; lanes 2 and 3) and pBM21 (1.0-kb *HpaI* deletion; lanes 4 and 5) were used in panels A and B, respectively. As a DNA probe, ^{32}P-labelled pBM20 was used. Size markers are indicated in kilobases.

Plasmids pBM40 and pBM45 were transformed to ST11, and erythromycin-resistant transformants were isolated. The transformation frequencies are indicated in Table 2. Individual transformants were analyzed by Southern analysis, and genomic plasmid integrations resulting in cointegrates similar to those described above were found. To resolve the cointegrate structures and to select for the remaining integrated *cat* gene (Fig. 6), erythromycin-resistant transformants were first grown under nonselective conditions as described above and then used to inoculate MSK broth containing 10 μg of chloramphenicol per ml. Independent erythromycin-sensitive, chloramphenicol-resistant cells were isolated from these milk cultures, and their genomic DNAs were analyzed by Southern blots and found to be identical. A representative culture, named ST11-Cat, was chosen for further analysis.

Southern blot and restriction maps of ST11-Cat and its preceding cointegrate are shown in Fig. 9. The 2.9-kb *ClaI* fragment of ST11 (lane 1), containing the *lacS* and *lacZ* junction, was increased to 3.5 kb in ST11-Cat (lane 2). Hybridization of the same blot with a probe containing only *cat* gene DNA revealed that this 3.5-kb fragment contained the integrated gene (lane 8). Hybridization with labelled pUC-838 demonstrated the absence of vector DNA in ST11 and ST11-Cat (lane 4 and 5). Furthermore, hybridizations with the different probes confirmed the structure of the cointegrate: the 4.7- and 3.8-kb fragments contained the integrated vector (lane 6), of which the 4.7-kb fragment carried the integrated *cat* gene (lane 9). The results presented were substantiated by similar hybridization experiments with genomic *PstI*, *BglII*, and *NdeI* digests.

Stability of the integrated *cat* gene. To investigate the genetic stability of the integrated *cat* gene, ST11-Cat was grown for 150 generations in the absence of chloramphenicol in MSK or M17 broth supplemented with lactose or sucrose. Cultures were then plated onto M17 agar plates and replica plated onto M17 agar plates containing chloramphenicol. All tested colonies were able to grow on the selective plates, indicating that the *cat* gene was stably maintained on the bacterial genome.

Expression and regulation of the integrated *cat* gene. The lactose transport (*lacS*), β-galactosidase (*lacZ*), and chloramphenicol acetyltransferase (*cat*) activities of ST11 and ST11-Cat grown on lactose, sucrose, and sucrose with galactose were determined (Table 3). Upon growth of the bacteria on lactose, the genes of the lactose operon were clearly expressed. The same was true for growth of the cells on sucrose in the presence of galactose, which induces the operon but cannot be fermented. Without galactose, only weak expression of the genes was observed. These results are in agreement with earlier studies of sugar utilization and inducible β-galactosidase activity of *S. thermophilus* (11, 33, 35, 48).

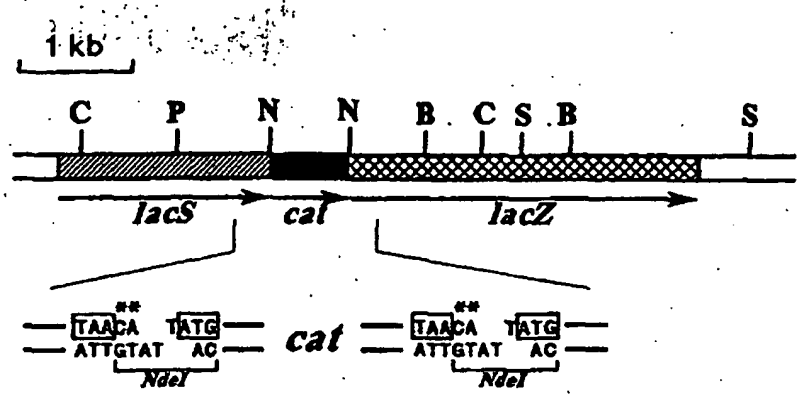

FIG. 8. Restriction map of the lactose operon containing the *cat* gene. Hatched boxes and arrows indicate the *lacS*, *cat*, and *lacZ* genes. Abbreviations for restriction sites: B, *Bgl*II; C, *Cla*I; N, *Nde*I; P, *Pst*I; S, *Spe*I. Boxed codons represent start and stop codons of the corresponding genes. Asterisks indicate the modified nucleotides of the intergenic region. Brackets underneath the DNA sequence point to the newly generated *NdeI* sites.

The results showed that ST11 grown on lactose exhibited moderately higher galactoside counterflow and β-galactosidase activities than cells grown on sucrose with galactose. Integration of the *cat* gene into the lactose operon did not affect the *lacS*-mediated counterflow activity, whereas the β-galactosidase activity was reduced to about 10%. Similar results were obtained for cells grown on lactose and on sucrose and galactose, irrespective of whether the cells were harvested in the exponential or stationary phase of growth (data not shown). This indicates that transcription from the *lac* promoter is not significantly affected upon integration of the *cat* gene into the operon.

The chloramphenicol acetyltransferase activities of ST11-Cat grown on lactose or on sucrose and galactose paralleled the respective β-galactosidase activities. The same was true for cells grown on sucrose without the addition of galactose. This indicates that both genes are expressed and regulated in the same way by the same genetic control elements.

DISCUSSION

In this study, we demonstrated Campbell-like integrations (4) into the genome of *S. thermophilus* of nonreplicating plasmids carrying small stretches of homologous genomic DNA that lead to the formation of cointegrates. All transformants analyzed showed genomic integration of the plasmid at a homologous locus. Thereby, a homologous region of between 0.7 and 0.9 kb was sufficient for integration and subsequent resolution. It is interesting that integration of linearized plasmids was more efficient than that of circular ones, similar to what was previously observed with yeasts (31). The increased recombination activity could be due to an induced DNA repair system upon the presence of free DNA ends in the bacterial cell. However, more work would be necessary to substantiate this hypothesis.

We observed that plasmids carrying lesions in the *lacZ* gene preferently integrated into the genome such that the functional copy of the gene was maintained within the lactose operon and the deletion located downstream of the integrated plasmid (Fig. 3). This is true whether the cells are grown on sucrose or glucose and despite the presence of homologous regions of about equal size on both sides of the lesion, e.g., for pBM20 and pBM26. There is no obvious explanation for this observation, as we have shown that cells that do not express functional β-galactosidase are perfectly viable.

Upon integration and growth under selective conditions, plasmids were often found as cointegrates of copies repeated in tandem. This could be a consequence of multicopy integration as well as of an amplification process after integration, due to the selective pressure imposed on the erythromycin gene. Analysis of such cointegrates from independent integration events of pBM26, which carries an in vitro-generated deletion within the homologous stretch of DNA, showed amplification not only of the in vitro-modified repeat but also of the original, unmodified homologous repeat (Fig. 4). This observation points to the occurrence of amplifications via homologous recombination of flanking sequences after an integration of the plasmid has taken place. Depending on which region of homology was used for recombination, either the original or modified repeat was amplified. Similar amplifications on plasmids or genomes of antibiotic resistance genes are known to occur in other bacterial species as well (6, 51).

We have shown that plasmid integration with subsequent resolution of the cointegrate structure is a feasible way to delete or insert genes (Fig. 6). For instance, the original *lacZ* gene could be replaced with an in vitro-truncated copy. In a similar way, an entire gene could be integrated into the bacterial genome in a targeted manner. To demonstrate this, a promoterless *cat* gene was inserted into the lactose operon and expressed via the control elements of this operon (Table 3).

Upon integration of *cat* in front of the *lacZ* gene, expression of the latter was reduced to about 10% of the wild-type activity but was still controlled by the *lac* promoter of the operon. A possible explanation may be that the insertion of a heterogenic gene into the *lac* operon structure interferes with correct RNA folding and/or that unconventional codon usage may introduce deleterious pausing in the translation process. This may increase messenger instability and degradation of the 3'-terminal end of the RNA (1). In addition, there is a potential ribosomal binding site for the wild-type *lacZ* gene which overlaps the 3'-terminal end of the *lacS* gene (32). This ribosomal binding site is likely to be a potent translational recognition site, important for translational reinitiation and RNA stability, which now serves for high-level expression of the *cat* gene. No similar site is present at

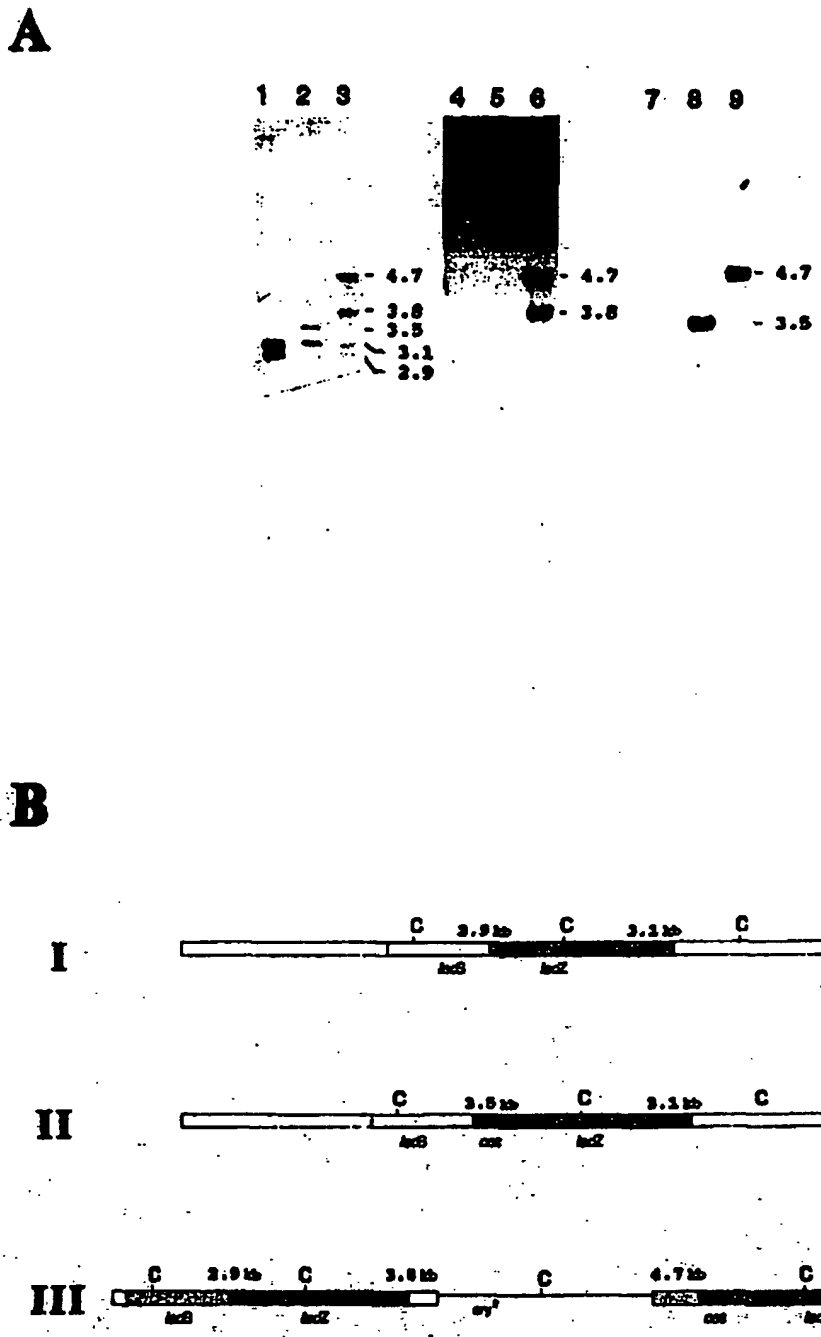

FIG. 9. Southern blots of the genomic integrated *cat* gene. (A) Hybridization blots with genomic *ClaI* digests of ST11 in lanes 1, 4, and 7; ST11-Cat in lanes 2, 5, and 8; and the corresponding pBM45 cointegrate in lanes 3, 6, and 9. Lanes 1 to 3 were hybridized with total pBM45 DNA, lanes 4 to 6 were hybridized only with *E. coli* vector DNA containing the erythromycin resistance gene, and lanes 7 to 9 were hybridized with an isolated *cat* gene probe. Fragment sizes are indicated in kilobases. (B) Schematic representation of the corresponding restriction maps of ST11 (I), ST11-Cat (II), and the pBM45 cointegrate (III). C, *ClaI* restriction sites. Fragment sizes are indicated in kilobases. Stippled, hatched, and solid areas represent the *lacS*, *lacZ*, and *cat* genes, respectively. The erythromycin resistance gene (ery^r) on the integrated plasmid DNA is indicated.

TABLE 3. Galactoside transport and enzymatic activities

Strain	Carbohydrate source[a]	Transport activity[b]	β-Galactosidase activity[c]	CAT activity[c]
ST11	Lactose	20.0 (100%)	6.40 (100%)	0.0
ST11	Sucrose, galactose	9.2 (46%)	2.35 (37%)	0.0
ST11	Sucrose	1.7 (9%)	0.41 (6%)	0.0
ST11-Cat	Lactose	20.0 (100%)	0.78 (100%)	0.80 (100%)
ST11-Cat	Sucrose, galactose	9.4 (47%)	0.30 (38%)	0.39 (48%)
ST11-Cat	Sucrose	1.7 (9%)	0.11 (14%)	0.14 (17%)

[a] Carbohydrate source indicates the sugar(s) which was present in growth medium (20 mM each).
[b] Transport was assayed as [^{14}C]TMG/TMG counterflow activity at 23°C. Initial rates of uptake were determined from the accumulation of radioactivity after 5 s of incubation (in triplicates). Activity is in nanomoles of TMG taken up per minute per milligram of total protein.
[c] β-Galactosidase and chloramphenicol acetyltransferase (CAT) activities are expressed in micromoles per minute per milligram of total protein.

the end of the *cat* gene, and this absence therefore may have caused the lower β-galactosidase activity. Despite the 10-fold-reduced β-galactosidase activity upon integration of *cat*, the cells are still able to grow normally on lactose- or sucrose-based medium. Apparently, ST11 possesses an excess of β-galactosidase over lactose transport activity, which allows down-expression of *lacZ* without causing an effect on the lactose metabolism. This aspect makes integration into the *lac* operon extremely suitable for efficient expression of foreign genes.

Integration of a heterologous gene into a vital operon of the *S. thermophilus* genome preserved its correct functioning. The foreign gene became a functional part of the operon and was regulated similarly. The integrated gene was stably maintained over 150 generations of cell growth in lactose- and sucrose-containing media. In addition, sporadic rearrangements or imperfect deletions of the gene would have destroyed the integrity or correct expression of the *lacS* and/or *lacZ* gene, which would have inhibited cell growth on lactose as the sole carbohydrate source. Hence, insertion of the gene between the *lacS* and *lacZ* genes ensures maintenance and expression of the integrated gene upon growth of the cells in their natural habitat, i.e., milk, without the necessity of additional selection.

Finally, no heterologous DNA, except for that of the foreign gene to be expressed, is present in the modified bacteria. Hence, the presented methodology is useful as a stable gene expression system for *S. thermophilus* and may qualify in the future for an application with food. As other, native operons may be chosen for similar integrative gene expression, different levels of expression and possibilities for regulation may be pursued.

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